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Short communication

A potential therapeutic target for regulating osteoporosis via suppression of osteoclast differentiation

Qin Sun[a], Boran Zhang[a], Wei Zhu[a], Wei Wei[a], Jingzhi Ma[a,*], Franklin R. Tay[b,*]

[a] Department of Stomatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] College of Graduate Studies, Augusta University, Augusta, GA, USA

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ABSTRACT

Objectives: Osteoclast differentiation is regulated by transcriptional, post-transcriptional and post-translational mechanisms. Micro-ribonucleic acids (miRNAs) are 20–24 nucleotides long non-coding RNAs involved in post-translational regulation of gene expressions during osteoclast differentiation. The objective of the present study was to investigate the role played by the miRNA, miR-338-3p, in osteoclastogenesis.

Methods: Osteoclastogenesis was induced in murine RAW264.7 cells using M-CSF and RANKL. The differentiated cells were harvested at designated times for TRAP staining and detection of designated gene expressions. A synthetic miR-338-3p mimic or its inhibitor was transfected into RAW264.7 cells prior to the induction of osteoclastogenesis. The effects of mimic or inhibitor on osteoclastogenesis were examined by qRT-PCR and TRAP staining. Bioinformatic analysis and luciferase activity were performed to identify the relationship between miR-338-3p and the transcription factor MafB. The miR-338-3p mimic and MafB siRNA were co-transfected into RAW264.7 cells to evaluate the cross-talk between miR-338-3p and MafB.

Results: miR-338-3p was increased significantly during osteoclast differentiation. Overexpression of miR-338-3p promoted osteoclastogenesis while its inhibition had the opposite effect. Bioinformatic analysis and dual luciferase assays indicated that miR-338-3p targeted MafB to repress its gene expression. MafB knockdown by RNA silencing blocked the promotional effect of miR-338-3p on osteoclast differentiation.

Conclusion: Because miR-338-3p is crucial for osteoclastic differentiation via targeting of the transcription factor MafB, inhibition of this miRNA represents a potential strategy for modulating osteoporosis in an aging population.

Clinical significance: Understanding the role played by miR-338-3p in osteoclast differentiation bridges the gap between the pathogenesis of osteoporosis and the quest for novel therapeutics to reduce the risk of bone fracture associated with this global disease.

1. Introduction

Bone homeostasis is incumbent upon the orchestrated functions of osteocytes, osteoblasts and osteoclasts [1–3]. Metabolic bone disorders such as osteoporosis, osteopenia and rheumatoid arthritis-related bone destruction occur when osteoclastic bone resorption exceeds osteoblastic bone formation. Unconstrained increase in bone catabolism results in osteoporosis, a highly-morbid, globally-escalating systemic skeletal disorder in which reduction in bone mineral density and micro-architectural deterioration of bone tissues increase the risk of bone fragility and fracture [4]. Osteoporosis-related fractures increases the mortality rate of the elderly [5,6]. To date, no effective strategies are available for preventing and treating this type of fracture [7].

Osteoclasts, the only cell type capable of bone resorption in the human body, are multinucleated cells derived from hematopoietic stem cells or monocyte/macrophage progenitor cells [8]. Unlike lymphocytes and macrophages that are destined to remove foreign bodies, osteoclasts are involved in the destruction of host tissues. This activity may be perceived as a physiologic auto-immune reaction in response to the need for bone remodeling. Differentiation of osteoclasts from hematopoietic precursors is a complex, multi-step process [9] regulated by a plethora of cytokines, growth factors and hormones, including macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL), sex hormones and parathyroid hormone [10,11]. Some of transcription factors involved in osteoclastogenesis have been identified, such as V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (transcription factor MafB), nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and microphthalmia-

* Corresponding authors.
E-mail addresses: majingzhi2002@163.com (J. Ma), ftay@augusta.edu (F.R. Tay).

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associated transcription factor (MitF) [12–14]. Nevertheless, the precise molecular mechanism of osteoclast differentiation remains obscure.

Micro-ribonucleic acids (miRNAs) are short, 20–24 nucleotides long, endogenously non-coding small RNAs that act as post-transcriptional regulators of gene expression via binding to the 3′-untranslated region (UTR) of target mRNAs to repress their translation [15]. They play important roles in cell differentiation, proliferation and self-renewal by providing an epigenetic mechanism for modulating gene expression in many homeostatic processes and pathological conditions [16]. A variety of miRNAs are involved in osteoclast differentiation [17]. For example, miR-503 in CD14+ peripheral blood mononuclear cells regulate osteoclast formation by targeting RANK [18]. By targeting NFATc1, miR-124 plays a significant role in regulating osteoclast differentiation [19]. The miRNA miR-34c promotes osteoclast differentiation by targeting leucine-rich repeat-containing G-protein coupled receptor 4, which is associated with the signalling of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and glycogen synthase kinase 3-β [20].

Although miRNAs are involved in bone remodelling and bone diseases [21], only a few microRNAs are concomitantly involved in osteogenesis and osteoclastogenesis [22]. To date, there is no information indicating that a specific microRNA is involved in the balance between osteoblast and osteoclast differentiation. For example, miR-21 and miR-26a were found to modulate the activities of osteoblasts and osteoclasts [21]. Although miRNAs serve as biomarkers and potential therapeutic targets for diseases [23], clinical research on these aspects is still at its infancy. No specific microRNA has been identified as the most optimal therapeutic target for osteoporosis [24]. The miRNA miR-338-3p functions as a negative regulator of osteoblast differentiation in bone marrow stem cells via epigenetic modulation of its target genes Runt-related transcription factor-2 (*Runx2*) and fibroblast growth factor receptor-2 (*Fgfr2*) [25]. This miRNA potentially modulates osteoporosis via its effect on osteoblasts [26]. Compared with normal tissues, miR-338-3p expression increases significantly in osteosarcoma [27]. These data suggest that miR-338-3p is involved in diseases of bone homeostasis. Accordingly, the present study tested the hypothesis that miR-338-3p suppresses the anabolic component of bone homeostasis by modulating a target transcription factor involved in osteoclastogenesis. Successful validation of this hypothesis paths the way for future research on the use of miR-338-3p as a biomarker and therapeutic target in preventing osteoporosis.

2. Materials and methods

2.1. Cell cultures and osteoclast differentiation

Murine RAW264.7 cells (American Type Culture Collection, Rockville, MD, USA) were used as a model for osteoclast differentiation. These cells were cultured in alpha-minimal essential medium containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Osteoclast differentiation was conducted by seeding cells on six-well plates in osteoclast differentiation medium. The latter consisted of 10% FBS, supplemented with 50 ng/mL recombinant mouse-RANKL and 30 ng/mL recombinant mouse-M-CSF (both from R&D Systems, Minneapolis, MN, USA). Differentiated cells were harvested at designated times for miRNA and mRNA extraction, as well as tartrate-resistant acid phosphatase (TRAP) staining.

Human embryonic kidney-derived, highly-transfectable 293T cells (Invitrogen, Carlsbad, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, penicillin and streptomycin (Invitrogen) at 37 °C in 5% CO_2 atmosphere prior to transfection.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from differentiated RAW264.7 cells at designated time periods (0–9 days) using the miRNeasy mini kit (Qiagen, Valencia, CA, USA). The miScript SYBR Green PCR kit (Qiagen) was used for analysis of miRNA expression. Small nuclear RNA (*U6 snRNA*) was used for miRNA normalisation. Primers for *miRNA-338-3p* and *U6 snRNA* were purchased from Qiagen. A SYBR Green PCR kit (Takara, Tokyo, Japan) was used for detection of mRNA expression. Primer sequences for the genes examined are listed in Supplementary Table I. Glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) was used for normalisation of mRNA measurements. Experiments were conducted in triplicate. Gene expression ratios were presented as means and standard deviations based on the results of three independent measurements from each experiment. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.3. Western blot

After quantification with BCA Protein Assay Kit (Pierce Biotech, Rockford, IL, USA), protein samples were analysed with sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes (Roche Diagnostics GmbH, Mannheim, Germany). After blocking with 5% skim milk, protein-containing membranes were incubated with anti-MafB antibody (1:1000; Abcam, Cambridge, MA) or anti-GADPH antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C and secondary anti-rabbit horseradish peroxidase-conjugated antibody. Antibody-antigen complexes were detected using chemiluminescent ECL reagent (Pierce Biotech, ThermoFisher Scientific, Waltham, MA, USA).

2.4. TRAP staining

Staining was performed with an acid phosphatase kit (MilliporeSigma, St. Louis, MO, USA). The cultured cells were fixed in fixative solution for 30 s at ambient temperature. After rinsing and addition of TRAP staining medium, the cells were incubated at 37 °C and protected from light for 1 h. Staining was performed in triplicate. Images of TRAP-positive multinuclear cells were recorded using an inverted phase-contrast microscope (IX41, Olympus, Japan).

2.5. Modulation of miR-338-3p

Transfection of miRNA mimics or inhibitors is a technique used to identify the targets and roles of specific miRNAs. Sequence-specific oligonucleotide transfection was performed using synthetic miR-338-3p mimic/inhibitor (Qiagen) to up-regulate/down-regulate the expression of the miRNA. These agents were transfected into RAW264.7 cells at a final concentration of 50 nM using lipofectamine 2000 as transfection reagent (Invitrogen). Transfection efficiency was monitored using plasmid encoding green-fluorescent protein (GFP; Supplementary Methods I). The transfected cells were purified by magnetic-affinity cell sorting to minimise the effect of background contamination of non-transfected cells on gene expressions of the transfected cell subpopulation (Supplementary Methods II). The effect of the transfection agents on cell viability was examined by flow cytometry of the purified transfected cells (Supplementary Methods III). Gene expressions of the two versions of purified, non-GFP encoded transfected cells and the non-transfected negative control were examined with RT-qPCR after 48 h of incubation.

2.6. miRNA target prediction

The potential targets of miR-338-3p were predicted using TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/) databases. Only mRNAs predicted by both algorithms to have a strong likelihood of miRNA:mRNA interaction were selected for further experimental validation. That is, the Context++ score should be less than −0.2 and the Pct score should be more than

0.2 for TargetScan; the mirSVR score should be less than −0.5 and the PhastCons score should be more than 0.5 for miRanda. Target genes unrelated to regulation of osteoclast differentiation and function were not considered.

2.7. Dual luciferase reporter assay

The 3′-UTR sequences of MafB containing seed-matched wild-type or mutant-binding sites were cloned into the pEZX-MT06 dual-luciferase vector (GeneCopoeia, Rockville, MD, USA). Wild-type or mutant target clones were co-transfected into 293T cells with the miR-338-3p mimic, using Lipofectamine 2000 as transfection reagent. Cells transfected with MafB target clones only, without miRNA mimic, served as the negative control. The transfected cells were harvested after 48 h and lysed. Assays were performed with the dual luciferase reporter assay system (Promega, Madison, WI, USA). Data were generated by normalisation of firefly luciferase activity against *Renilla* luciferase activity. Co-transfection assays were conducted in triplicates.

2.8. Over-expression and knock-down of MafB

Over-expression of MafB was achieved by transfecting RAW264.7 cells with the over-expression plasmid pcDNA3.1@MafB (Sangon Biotech, Shanghai, China), using lipofectamine 2000 as transfection reagent. Cells transfected with the pcDNA3.1 empty vector was used as negative control. Knock-down of MafB RAW264.7 cells was performed using small interfering RNA (siRNA) against MafB (SiRNA@MafB, Sangon Biotech) or the corresponding sham siRNA (Sangon Biotech). After 48 h of post-transfection, cells were harvested for qRT-PCR to detect MafB mRNA expression. After incubation in induction medium for 3 additional days, a second batch of cells was harvested for qRT-PCR detection of the mRNA expression of osteoclast-related genes and for TRAP staining.

3. Statistical analyses

For each analysis, the data sets were analysed for their normality (Shapiro-Wilk test) and equal variance (modified Levene test) assumptions prior to the use of parametric statistical methods. Parametric data were expressed as means ± standard deviations. If those assumptions were not violated, data sets with more than three groups were analysed with one-factor analysis of variance (ANOVA) and Holm-Sidak pairwise comparison procedures. Data sets with two groups were analysed with the Student *t*-test. If the normality and equal variance assumptions were violated, the data were non-linearly transformed prior to the use of the aforementioned analyses. For examining the effect of MafB knock-down on the function of miR-338-3p, two-factor ANOVA was used to examine the effects of “miR-338-3p over-expression” and “siRNA knock-down of MafB”, and the interaction of those two factors on expression of osteoclastogenesis-related genes. Post-hoc comparisons were conducted using the Holm-Sidak procedure. For all testing procedures, statistical significance was pre-set at $\alpha = 0.05$.

4. Results

Compared with undifferentiated RAW264.7 cells (designated as D0), activation of the cells with RANKL and M-SCF resulted in statistically-significant up-regulation of osteoclastogenesis-specific genes *NF-kB*, *MitF* and *NFATc1* at each of the designated post-induction periods (3, 5, 7 and 9 days, designated as D3-D9; Fig. 1A–C). Whereas *miR-338-3p* gene expression was progressively up-regulated during osteoclastogenesis (Fig. 1D), expression *MafB*, a putative target for miR-338-3p, was progressively down-regulated during those test periods (Fig. 1E), with significant differences identified among all time-periods ($p < 0.05$). The multinucleated characteristic of differentiated osteoclasts was evident at around day 7 (D7) after TRAP staining (Fig. 1F).

The potential link between miR-338-3p and osteoclastogenesis, identified in Fig. 1D, was further validated by transfecting RAW264.7 cells with a synthetic miR-338-3p mimic or its inhibitor prior to the induction of osteoclastogenesis (Fig. 2A). Flow cytometry indicated that the transfection efficacy of the miRNA mimic was 73.2% and that of the miRNA inhibitor was 95.2%. The percentages of viable, non-apoptotic non-transfected cells, purified cell subpopulation transfected with the miRNA mimic, and purified cell subpopulation transfected with the miRNA inhibitor were 72.8%, 65.3% and 68.6%, respectively. After 3 days of induction, gene expressions of *NF-kB*, *MitF* and *NFATc1* were significantly up-regulated by the miR-338-3p mimic (Fig. 2B–D). In contrast, expression levels of these gene markers were significantly down-regulated after transfection with the miR-338-3p inhibitor (Fig. 2B–D). The number of TRAP-positive multinucleated osteoclasts increased in the miR-338-3p mimic group and decreased in the miR-338-3p inhibitor group, compared with the non-transfected negative control (Fig. 2E).

Computational sequence-based predictions enable rapid identification of putative miRNA targets [28]. Because gene expression levels of the transcription factor MafB were contrariwise to the up-regulations of miR-338-3p (Fig. 1E), bioinformatics were used to predict potential miRNA:mRNA interactions for miR-338-3p regulation of osteoclastogenic differentiation, and for minimizing false-positive rates, prior to further experimental validation. TargetScan and miRanda algorithms prioritized the *MafB* gene as the most likely target candidate for miR-338-3p interaction (Supplementary Table II). The 3′-UTR region of murine *MafB* contained a binding site with trailer sequence that was complementary with the seed sequence of miR-338-3p (Fig. 3A). The complementary trailer sequence was absent in the engineered miR-338-3p mutant. Transfection of osteoclast precursor cells with the miR-338-3p mimic resulted in significant suppression of *MafB* mRNA (Fig. 3B) and its protein levels (Fig. 3C), compared with the non-transfected negative control. Conversely, transfection of the miR-338-3p inhibitor resulted in significant over-expression of *MafB* mRNA (Fig. 3D) and its protein levels (Fig. 3E), compared with the negative control. The luciferase reporter assay conducted in 293T cells definitely confirmed that miR-338-3p targets MafB. As shown in Fig.3F, miR-338-3p over-expression in the wild-type MafB 3′-UTR resulted in significantly reduced luciferase activity, whereas mutation of the binding site of miR-338-3p eliminated this inhibitory effect.

Over-expression and inhibition of MafB was performed to clarify the role played by this transcription factor in osteoclastogenesis. Gene expression profiles are shown in Fig. 4 (top panel). Endogenous *MafB* expression was significantly increased after over-expression with the plasmid vector pcDNA3.1@MafB for 48 h (Fig. 4A). Osteoclastogenesis induction conducted after transfection of the over-expression plasmid for MafB resulted in significant down-regulation of *NF-kB*, *MitF* and *NFATc1* expressions (Fig. 4B–D). In contrast, *MafB* expression was significantly inhibited after siRNA-induced gene knock-down (Fig. 4E), with significant down-regulation of the expression of osteoclastogenesis-specific genes (Fig. 4F–H). A similar trend was also identified with TRAP staining for multinucleated osteoclasts (Fig. 4, bottom panel).

To identify the cross-talk between miR-338-3p and MafB during osteoclast differentiation, the miR-338-3p mimic was introduced into RAW264.7 cells after they were transfected with siRNA against MafB. Co-transfected cells were subsequently treated with RANKL and M-SCF to stimulate osteoclastogenesis. For *NF-kB*, *MitF* or *NFATc1*, gene expression was significantly affected by *miR-338-3p* over-expression ($p < 0.001$) and *MafB* knock-down ($p = 0.03$); the interaction of these two factors was also significant ($p < 0.001$). Post-hoc analysis revealed that *MafB* knock-down had significant, but opposite effects in cells with endogenously-produced miR-388-3p (sham) or those with over-expression of the miRNA (mimic) (Fig. 5, top panel); gene expressions were up-regulated in the sham-groups and down-regulated in the mimic-groups after *MafB* knock-down ($p < 0.05$). In the absence of *MafB* knock-down, transfection of the miR-338-3p mimic significantly

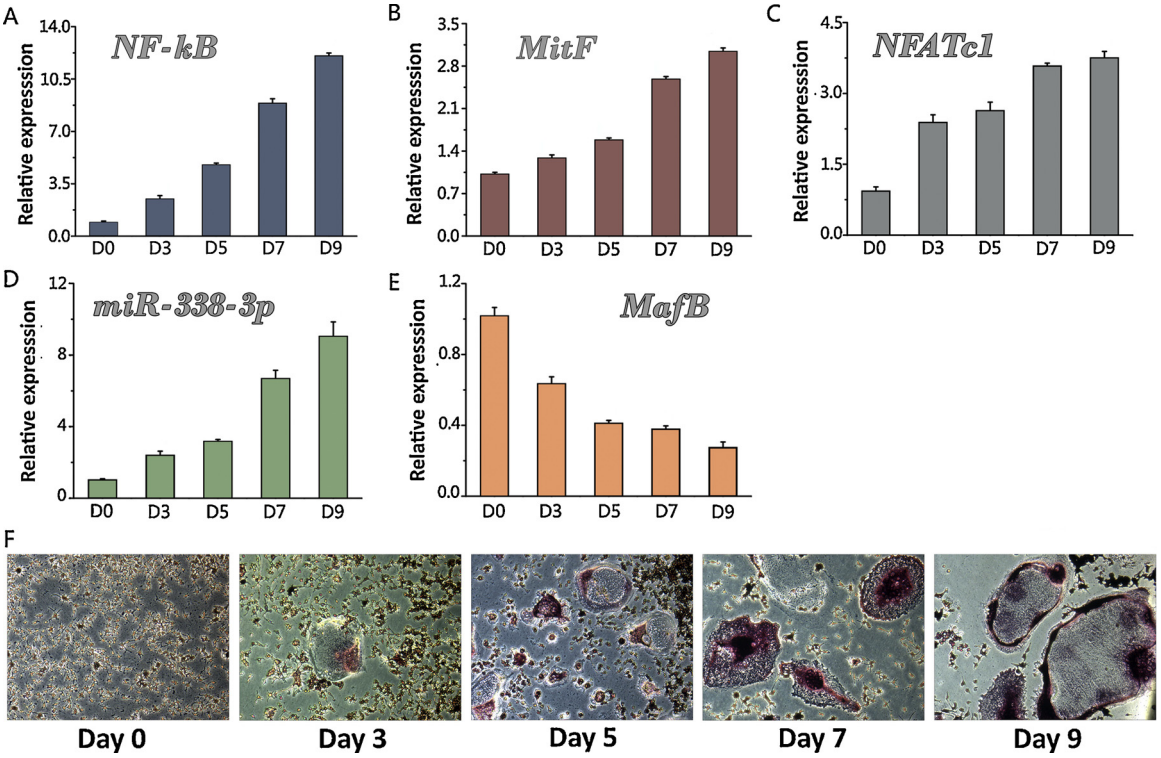

Fig. 1. Osteoclastic differentiation of RAW264.7 cells. **A-C**. Expression of osteoclast differentiation marker genes *NF-kB* (**A**), *MitF* (**B**) and *NFATc1* (**C**) at designated time-periods (0–9 days, designated as D0-D9). **D**. Expression of *miR-338-3p* levels at designated time-periods. **E**. Expression of *MafB* mRNA levels at designated time-periods. Data were normalized to *GADPH* (for mRNA) or *U6* (for miRNA) and presented as means and standard deviations. For each chart (n = 9), pairwise comparisons indicated that all groups are significantly different ($p < 0.002$ for all charts). **F**. TRAP staining performed at 0, 3, 5, 7 and 9 days.

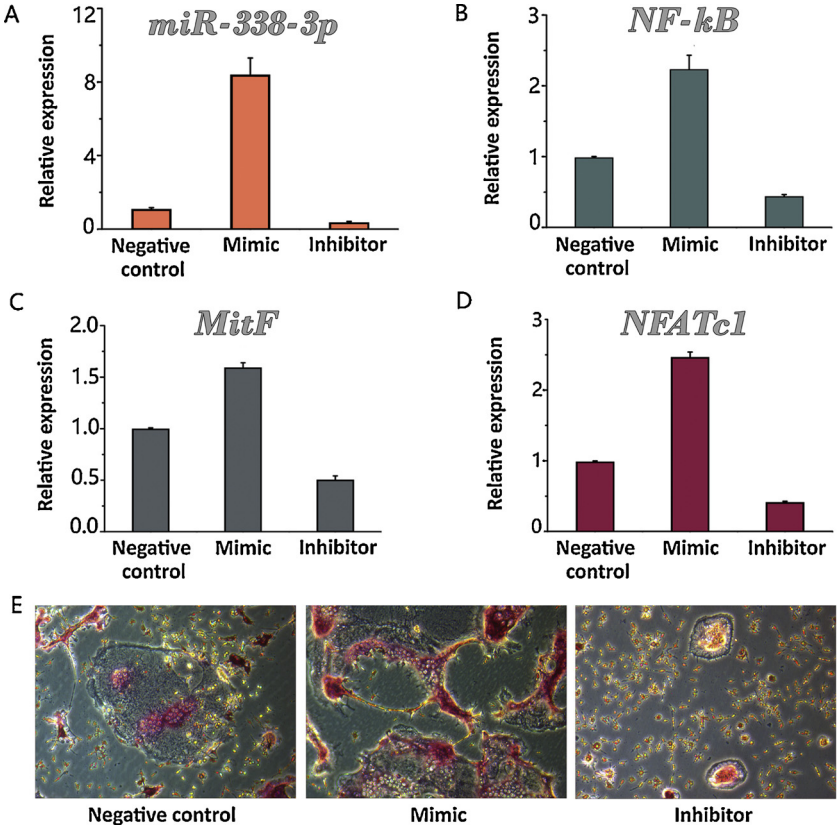

Fig. 2. MicroRNA-338-3p regulation of M-CSF and RANKL-induced osteoclastogenesis of RAW264.7 cells. **A**. RAW264.7 cells transfected with miR-338-3p mimic (mimic) or miR-338-3p inhibitor (inhibitor) to up-regulate or down-regulate *miR-338-3p* expression. Results were compared with non-transfected cells (negative control), **B-D**. Expression of *NF-kB*, *MitF* and *NFATc1* mRNA after induction of osteoclast differentiation for 3 days. Data were normalized to *GADPH* (for mRNA) or *U6* (for miRNA) and presented as means and standard deviations (n = 9). For each chart, pairwise comparisons indicated that all groups are significantly different ($p < 0.001$ for all charts). **E**. TRAP staining performed at day 3 of osteoclastic differentiation to identify TRAP-positive multinucleated cells.

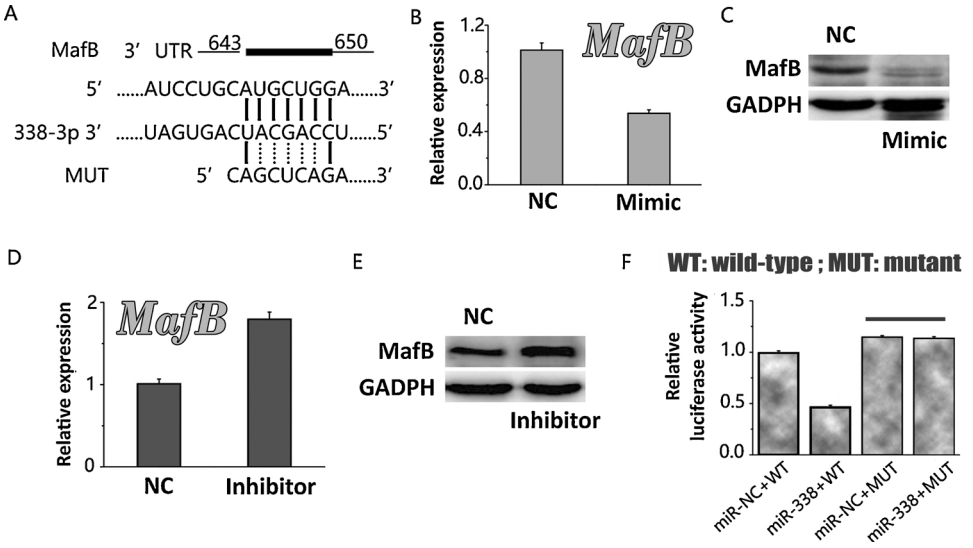

Fig. 3. Effect of miR-338-3p on MafB. **A**. Diagram of the 3′-UTR region of *MafB* mRNA illustrating the miR-338-3p binding site and the engineered mutation (MUT). **B**. mRNA and **C**. protein expressions of MafB in RAW264.7 cells transfected with miR-338-3p mimic (mimic), compared with non-transfected cells (NC). *GADPH* was used as internal controls. mRNA (**D**) and protein (**E**) expressions of MafB in RAW264.7 cells transfected with miR-338-3p inhibitor (inhibitor), compared with non-transfected cells (NC). *GADPH* was used as internal controls. For **B** and **D**, data were presented as means and standard deviations (n = 9). For each chart, the experimental group is significantly different from the negative control (NC; $p < 0.001$). **F**: Relationship between miR-338-3p and MafB examined with dual luciferase reporter system using non-transfected RAW264.7 cells (miR-NC) or miR-338-3p mimic-transfected RAW264.7 cells (miR-338) containing 3′-UTR sequences of MafB with seed-matched wild-type (WT) or mutant-binding sites (MUT). Data were presented as means and standard deviations (n = 6). Groups identified with the horizontal bar are not significantly different ($p > 0.05$).

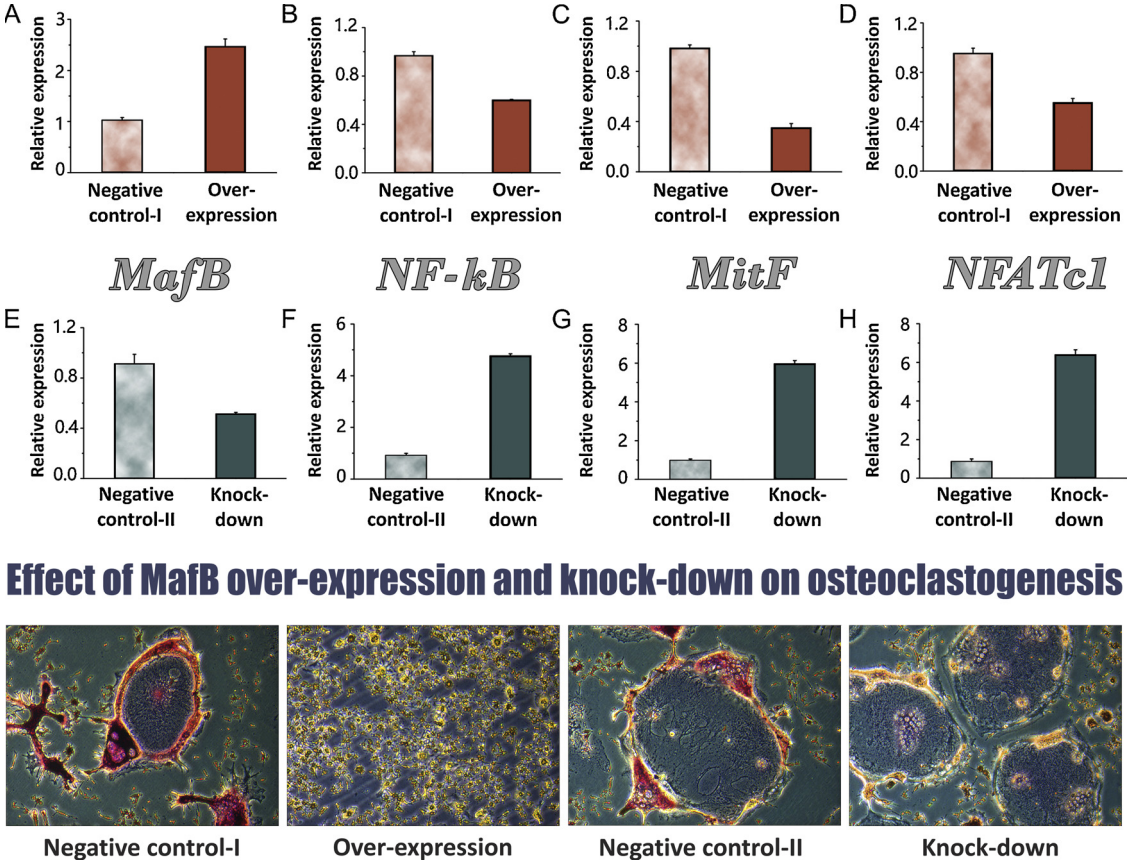

Fig. 4. Effects of MafB over-expression and knock-down on osteoclastogenesis. **Top panel**: MafB over-expression. **A**. *MafB* mRNA expression in RAW264.7 cells transfected with the over-expression plasmid pcDNA3.1@MafB (over-expression) or empty plasmid vector (negative control-I). **B-D**. Expression of osteoclast differentiation marker genes *NF-kB* (**B**), *MitF* (**C**) and *NFATc1* (**D**) in cells transfected with pcDNA3.1@MafB (over-expression) or the empty vector (negative control-I). Data are means and standard deviations (n = 9) for each chart in the top panel; the over-expression group is significantly different from negative control-I ($p < 0.001$ for all charts). **Middle panel**: MafB knock-down. **E**. *MafB* expression in cells transfected with siRNA against MafB (knock-down) and the corresponding negative control siRNA (negative control-II). **F-H**. Expression of *NF-kB* (**F**), *MitF* (**G**) and *NFATc1* (**H**) in cells transfected with SiRNA against MafB (knock-down) or the negative control SiRNA (negative control-II). Data were presented as means and standard deviations (n = 9) for each chart in the top panel; the knock-down group is significantly different from negative control-II ($p < 0.001$ for all charts). **Bottom panel:** representative images of TRAP-stained cells derived from the negative control-I, negative control-II, and cells after MafB over-expression or knock-down.

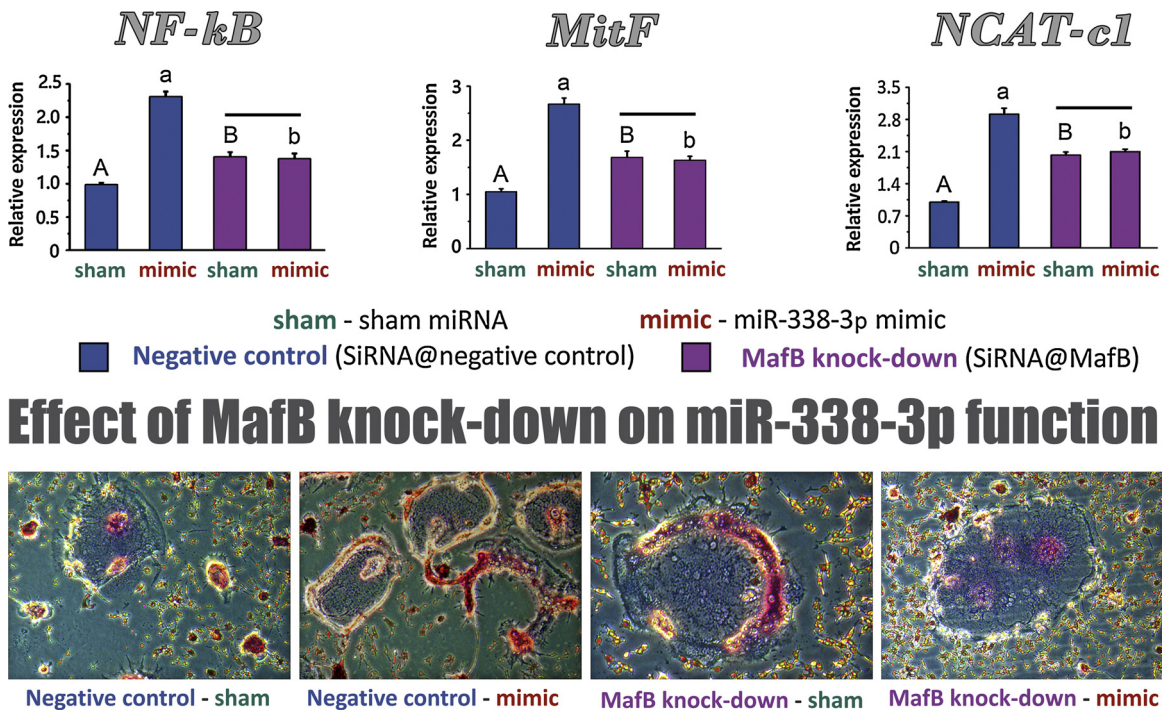

Fig. 5. Effect of MafB knock-down on miR-338-3p function. **Top panel**: Gene expression profiles of *NF-kB*, *MitF* and *NCAT-c1* after transfection of the miR-338-3p mimic or the corresponding sham miRNA (sham) into RAW264.7 cells with MafB knock-downed by siRNA against MafB (MafB knock-down) or cells with intact MafB (negative control). Data were normalized against GADPH and presented as means and standard deviations (n = 9). For the effect of miR mimic on gene expression in each chart, negative control columns (*blue*) or MafB knock-down columns (*purple*) connected with a horizontal bar are not significantly different ($p > 0.05$; post-hoc comparisons after two-factor ANOVA). For the effect of MafB knock-down on gene expression in each chart, sham columns labelled with the same upper-case letters and mimic columns labelled with the same lower-case letters are not significantly different ($p > 0.05$; post-hoc comparisons after two-factor ANOVA). **Bottom panel:** representative images of TRAP-stained cells derived from the four groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

up-regulated osteoclastogenesis-related genes ($p < 0.05$). Over-expression of miR-338-3p no longer promoted osteoclastogenesis after *MafB* knock-down ($p > 0.05$). That is, deletion of the target gene nullified the regulatory effects of miR-338-3p on osteoclast differentiation. These qRT-PCR results were supported by TRAP staining for multinucleated osteoclasts in the four groups (Fig. 5, bottom panel).

5. Discussion

Tools for evaluation of diseases associated with bone homeostasis in the clinical setting are scanty. Likewise, drug available for the treatment of osteoporosis are also limited. Bisphosphonates (e.g. alendronate) and RANKL inhibitors (e.g. denosumab) are commonly used for treatment of osteoporosis and reducing the risk of fracture. Long-term use of these drugs inhibits osteoblast and osteoclast functions simultaneously and may result in medication-related osteonecrosis of the jaw (MRONJ) after tooth extraction or surgical manipulation of the jaw bones [29]. Hence, investigation of the molecular mechanisms that mediate osteoclastic differentiation supports the development of diagnostic and treatment regimens for managing a highly-morbid bone disorder that adversely affects the quality of life of the affected individuals.

The precursor sequence of miR-338 is intronically-encoded within the apoptosis-associated tyrosine kinase (AATK) gene; spliced, mature forms of miR-338 are responsible for down-regulating genes that have a negative downstream effect on AATK expression [30]. Previous studies suggest that miR-338-3p promotes odontoblast differentiation by targeting Runx2 but plays a negative role in osteoblast differentiation [25,31]. Contrary to the finding that miRNA inhibits glucocorticoid-induced osteoclast formation through RANKL targeting [32], the expression level of miR-338-3p increased during M-CSF and RANKL-induced osteoclastogenesis of RAW264.7 cells in the present work. Over-expression of miR-338-3p increased the formation of TRAP-positive multinuclear cells and elevated the expression of osteoclast differentiation markers, while suppression of miR-338-3p achieved the opposite effects. These results suggest that miR-338-3p acts as a promotor of osteoclast differentiation.

Bioinformatics identified MafB, a member of the Maf family of basic leucine zipper transcription factors [33], as a putative target gene of miR-338-3p. The transcription factor is selectively expressed in the myeloid lineage of the hematopoietic system and is an inducer of monocyte differentiation. In the bone marrow, MafB is selectively expressed in monocytes and macrophages but not in other mature myeloid or lymphoid cells [34]. Lack of MafB in hematopoietic stem cells results in their self-renewal and proliferation, while MafB expression causes differentiation of these stem cells into macrophages via enhancement of their sensitivity to granulocyte colony-stimulating factor [35].

It was previously reported that MafB suppresses RANKL-induced osteoclastogenesis by interfering with the DNA-binding domains of transcription factors c-Fos, Mitf, and NFATc1, thereby inhibiting transactivation of NFATc1 and osteoclast-associated immunoglobulin-like receptor (OSCAR) [14]. Results from the present study validated that MafB inhibits osteoclast differentiation; knockdown of MafB promoted osteoclastogenesis while overexpression of MafB decreased this process (Fig. 4). The effect of miR-338-3p during osteoclast differentiation was blocked after deletion of MafB (Fig. 5), which is evincive of the regulatory relationship of miR-338-3p on MafB. Taken together, the present data suggests that miR-338-3p controls osteoclast differentiation by binding to MafB and silencing the related signalling pathways.

An miRNA may have multiple target genes, or one mRNA may be targeted by several miRNAs [36]. As a transcription factor, MafB is

necessary but not sufficient by itself for osteoclast differentiation. It must bind to other factors to control osteoclastogenesis [14]. In addition, multiple miRNAs may bind to the same mRNA to act cooperatively in reducing mRNA translation [37]. A recent study reported that miR-148a regulates osteoclast differentiation by targeting MafB [38]. Hence it is possible that miR-338-3p collaborates with other miRNAs to control osteoclast differentiation.

Cells derived from osteoblast and osteoclast lineages communicate with each other through cell-cell contact, diffusible paracrine factors and interaction with immune cells in the bone marrow microenvironment, including adipocytes, T cells and macrophages [39]. These intercellular cross-talks are essential for bone remodelling and mineral homeostasis. MicroRNA represent a sophisticated level of gene regulation that coordinates a broad spectrum of biological processes in the regulation of bone remodelling [40]. However, the control of miRNAs in the cross-talk between osteoblasts and osteoclasts remains to be addressed. The role of miR-338-3p in the regulation of both osteoclasts and osteoblasts suggests that this miRNA may be such a mediator. Accordingly, therapeutic blockage of miR-338-3p may be helpful in controlling osteoporosis and other bone metabolic disorders. The exact molecular mechanisms of miR-338-3p in mediating cross-talk between osteoblasts and osteoclasts should be further explored by performing complementary in vitro and in vivo studies.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdent.2019.01.015.

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